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Fine Structure and Immunogoldlabeling of Crystalline Inclusion Bodies in Mitochondria

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The distribution of respiratory chain complexes in beef heart and human muscle mitochondria has been explored by immunoelectron microscopy with antibodies made against beef heart mitochondrial proteins in conjunction with protein A colloidal gold (12nm particles). The antibodies used were made against NADH-conezyme Q reductase(complex I), ubiquinol-cytochrome-c-oxidoreductase (complex III) and cytochrome-c-oxidase(complex IV).

Labeling of beef heart tissue with any of these antibodies gave gold particles randomly distributed along the mitochondrial inner membrane. The labeling of muscle tissue mitochondria from a patient with a mitochondrial myopathy localized by biochemical analysis to complex III was quantitated and compared with the labeling of human control muscle tissue mitochondria.

Four kinds of morphological changes in the mitochondrial fine structure in the myopathy patient tissue have been found; paracrystalline inclusions consisting of densely packed multilamellar structures, globular crystalline inclusions with high electron density, multilamellar structure inclusion body(compactly and irregularly arranged concentric whirl shaped cristae) and golbular cyrstalline inclusions located in the center of the whirl shaped cristae.

Compex I and cytochrome-c-oxidase antibodies reacted to the same level in the mitochondria containing the crystalline inclusions and control mitochondria. Antibodies to complex III reacted very poorly to the mitochondria containing the crystalline inclusions but strongly to control mitchondria. The globular crystalline inclusions in the mitochondria are not reacted antibodies to respiratory chain complexes.

KEY WORDS: Inclusion bodies, Immunogoldlabeling

The morphological changes in mitochondria of skeletal muscles are reported. Recently, biochemical and clinical studies have also been done in these mitochondria at the molecular level (Shy and Gonatas, 1964, Kennaway *et al.*, 1984; Sengers *et al.*, 1984; DiMauro *et al.*, 1985).

The mitchondria containing electron dense inclusions in the matrix of the inner and outer mitochondrial membranes or with whirl shaped cristae are aggregated in subsarcolemmal regions of the muscle fibre.

Biochemically they have a deficiency in several components of ubiquinol-cytochrome-c-oxidoreductase (Kennway *et al.*, 1984; Behbehani *et al.*, 1984; Kim *et al.*, 1987) and cytochrome-c-oxidase of respiratory chain (Sengers *et al.*, 1984).

The electron dense inclusions in the mitchondria are composed of protein subunits of the enzyme complex of the respiratory chain (Farrants *et al.*, 1986) and structural changes in the mitochondria are accompanied by disorders in the respiratory chain or oxidation-phosphorylation coupling (DiMauro *et al.*, 1985).

We describe here ultrastructural studies and immunogold-labeling in the mitochondria of beef heart tissue, mitochondria myopathy patient tissue and normal human skeletal muscle tissue aimed at determining the relationship between the electron

dense inclusions in the mitochondria and the enzymes of the respiratory chain using antibodies against bovine heart mitochondria complexes I, III and IV.

Materials and Method

Tissue Preparation

The beef heart muscles, control and mitochondrial myopathy patient skeletal muscles were used as the experimental materials and all of them were stored as frozen tissue at −70°C.

The samples were fixed in 1% paraformaldehyde-1% glutaraldehyde in 0.12M phosphate buffer for 2 hr at 4°C, rinsed with the same buffer and secondary fixed in 2% osmium tetroxide for 1 hr at 4°C. The samples were then dehydrated in a graded series of ethanol at −20°C in a deep-freezer and then infiltrated with Lowicry HM20 at −20°C. Polymerization was performed by illuminating the samples under UV light (15 watt, 2 lamps) at −20°C according to the modified technique of Roth *et al.* (1981a).

Thin sections were cut on an ultramicrotome with glass knives and silver color ribbons were mounted on nickel grids for immuno-cytochemical goldlabeling. After immunization, grids were stained with 2.5% uranyl acetate and lead citrate and observed with a Philips 300 electron microscope.

Antibodies

Beef heart ubtiquinol-cytochrome-c-oxidoreductase (complex III or bc_1 complex) and cytochrome-c-oxidase (complex IV) were prepared according to the method of Capldi and Hayashi(1972) and then dried under a stream of nitrogen. Each protein(1 mg) was dessolved in distilled water mixed with an equal volume of Freunds complete adjuvent and injected into rabbits. Booster injections were also done 2 times at intervals of 2 weeks after the 1 st injection. The blood was then collected from the rabbits 2 weeks after the last injection and purified by ammonium sulfate. anti-NAD-ubiquinon reductase(complex I) antibody was the generous gift of Dr. J. Here and R. Hall, Oregon Health Sciences University, Protland, Oregon U.S.A..

Immunocytochemical Goldlabeling

The grids with sections were rinsed with distilled water for 10 min and etched in saturated sodium metaperiodate for 30 min. Grids were then washed for 5 min in distilled H_2O followed by 5 min. in buffer(Tris-HCl pH 8.2). They were then incubated for 1 hr at room temperature with antibody diluted 1 : 5 in buffer. Following buffer washing the grids were then incubated in a 1 : 20 dilution of 12 nm protein-A-colloidal gold solution prepared according to the method of Roth *et al.* (1981b) and Slot and Geuze(1985) for 1 hr at room temperature. The grids were then washed in buffer and distilled water.

The density of goldlabeling for each antibody was calculated from ten separate areas of sections containing mitochondria on ten different muscle tissue pieces.

Results

Ultrastructure

The electron dense enlarged cristae is composed of the inner membrane of mitochondria and a lot of glycogen accumulates in the sarcoplasm of the muscle of the mitochondrial myopathy patient (Fig. 1).

The mitochondria containing the paracrystalline inclusion are elongated in length and the paracrystalline inclusion is made up of several multilamellar structures and is densely packed (Figs. 2 & 3). Most of the mitochondria are aggregated in the sacroplasm under the sarcolemma. A few of them have irregularly concentrated whirl shape cristae and are larger than the other mitochondria (Fig. 4). The globular crystalline inclusions which are very electron dense usually appear in normally sized mitochondria which remarkabely have a few residual cristae or degenerated cristae (Figs. 4 & 5). The globular (Figs. 4, 5, 12 & 15) and are also observed in the center of irregularly concentrated whirl shape cristae (Fig. 13).

Morphologically changes mitochondria therefore have 4 kinds of ultrastructural characteristics; paracrystalline inclusions, globular crystalline inclusions,

Table 1. Quantitative analysis of the labeling density of the different respiratory chain antibodies in mitochondria and muscle fibre of different tissue(gold particles/0.1 μm^2 ±SD).

		Complex I	Complex III	Complex IV
Beef heart	mi.	3.44(±0.11)	36.84(±3.29)	38.749±6.21)
muscle tissue	mu.	0.85(±0.29)	2.43(±0.88)	1.12(±0.39)
Human skeletal	mi.	2.01(±0.46)	10.86(±0.74)	13.57(±1.82)
muscle tissue	mu.	0.39(±0.28)	2.069±0.44)	1.46(±0.65)

*mi; mitochondria mu; muscle

*The density of gold labeling for each antibody was calculated from ten separate areas of sections containing mitochondria on ten different muscle tissue pieces.

compactly concentrated whirl shape cristae and globular crystalline inclusions containing whirl shape cristae.

Immunogoldlabeling

Complex I(NADH-Ubiquinon reductase) antibody: The density of gold particles when labelled by complex I antibody is 3.44(0.11)/0.1 μm^2 in beef heart mitochondria and 2.01(0.46)/0.1 μm^2 in human skeletal muscle mitochondria(Table 1; Figs. 6 & 7). Very simular results are also obtained with the quantitative analysis between the beef and human tissue. All of the mitochondria are densely labeled on the cristae of both tissue (Figs. 6 & 7). Labeling over the myofibrils however is 0.85(0.29)/0.1 μm^2 on beef heart and 0.39(0.28)/0.1 μm^2 on human skeletal muscle. Although some nonspecific labeling occurs over the fibrils of both tissues, it is negligible in comparison to the high labeling over the mitochondria.

Goldlabeling with complex I for mitochondria containing electron dense globular crystalline inclusions appears to be similar to that of human skeletal muscle mitochondria. However no goldlabeling is found in globular cyrstalline inclusions(Figs. 6, 7 & 12).

Complex III (Ubiquinol-Cytochrome-c-oxidoreductase) antibody: The quantitation of goldlabeling in beef heart for complex III atibody gives a density of 36.84(3.29)/0.1 μm^2 in mitchondria and 2.43(0.88)/0.1 μm^2 in myofibrils(Table 1, Fig. 8). In the case of human skeletal muscle, it is 10.86(0.74)/0.1 μm^2 over mitochondria and 2.06(0.44)/0.1 μm^2 over myofibrils. Therefore these results confirm that specific immunoreactions are found in human mitochondria against beef heart complex III antibody. This also indicates a tissue specity because the density of gold particles on beef heart mitochondria is twice as high as that on human skeletal muscle mitochondria.

The globular inclusions in the mitochondria are not labeled by complex III antibody (Figs. 12, 13 & 14).

Complex IV (Cytochrome-c-oxidase) antibody: For the labeling of beef heart by complex IV antibdy, 38.74(6.21)/0.1 μm^2 are found over mitochondria and 1.12(0.39)/0.1 μm^2 over the myofibrils. For human skeletal muscle, the value is 13.57(1.82)/ 0.1 μm^2 in mitochondria and 1.46(0.65)/0.1 μm^2 in myofibrils. Nonspecific Immunoreactions are observed in both types of myofibrils and their quantitative value is similar to the results of complex III antibody. Tissue specificity clearly appears in the mitochondria of both tissues (Table 1, Figs. 10 & 11). The crystalline inclusions again are not labeled by complex IV, however the number of gold particles is almost the same when compared to normal mitochondria, mitochondria containing crystalline inclusions and whirl shaped mitochondria.

Discussion

The abnormalities in the fine structure and metabolism of mitochondria have already been reported in mitichondrial myopathy(Jerusalem *et al.*, 1971; bonilla *et al.*, 1975; Heine *et al.*, 1979; Mammersen *et al.*, 1980).

In morphorogical studies with the electronmicro-

scope, the aggregated mitochondria are commonly located under the salcolemma of the muscle cell. Remarkably they contain inclusions such as paracrystalline inclusions and globular inclusions-(DiMauro *et al.*, 1985) or have circular cristae(Behbehani *et al.*, 1984). The mitochondria appear as numerous ragged red fibers under the light microscope(Olson *et al.*, 1972).

Biochemically the specific changes of alternations in enzymatic activity of the mitochondria in mitochondrial myopathy have also been studied-(Kennaway *et al.*, 1984; DiMauro *et al.*, 1985).

Four kinds of morphological changes in the mitochondrial fine structure have been found; paracrystalline inclusions consisting of densely packed multilamellar structures, globular crystalline inclusions with high electron density, compactly and irregularly arranged concentric whirl shaped cristae and globular crystalline inclusions located in the center of the whirl shaped cristae.

Immunogoldlabeling was attempted on these abnormal mitochondria using antibodies against beef heart respiratory enzymes. We first tried beef heart muscle tissue with antibodies from complex I, III and IV.

Specific labeling was observed and the number of gold particles is similar to the abundance of these respiratory chain components in the mitochondrial inner membrane of beef heart as determined by biochemical studies (Capaldi, 1982). This confirms that the distribution and amount of the respiratory enzymes could be visualized by the immunogoldlabeling method.

Normal human skeletal muscle was also labeled with the same antibodies in order to identify which specific immunoreactions appear in human tissue with beef enzyme antibodies. In our results, the gold particles specifically bind to the control human mitochondria in the ratio of 1 : 5 : 10 for antibody to complex I, III, and IV, respectively. This result is similar to that for beef heart tissue. However, although complex IV isolated from human muscle has the same unit composition as the beef heart enzyme (Heare *et al.*, 1980), the number of gold particles on control human mitochondria is half that of beef heart mitochondria for complexes III and IV.

We suggest that tissue specificity seems to have occured in human mitochondria for antibody against the beef mitochondrial enzymes. The labeling by complex I antibody of the control mitochondria is similar to the mitochondria with the crystalline inclusions. But the globular crystalline inclusions are not labeled by complex I antibody. This result confirms that the mitochondria containing globular crystalline inclusion lack the activity of complex I. biochemically a deficiency of complex I (Kerr *et al*, 1980), complex III (Darley-Usmar *et al.*, 1983; Kennaway *et al.*, 1984; Behbehani *et al.*, 1984) and complex IV (DiMauro *et al.*, 1981; Willems *et al.*, 1977) in the mitochondria of regged fiber myopathy are detected by spectral analysis measurements of enzyme activity and immunoblotting. Kim *et al.*(1987) have proposed the diagnosis of mitochondrial myopathies involving the respiratory chain using the immunogoldlabeling method. Based on the quantitation of labeled gold particles compared to control muscle mitochondria, complex III is not assembled in the mitochondria of patient muscle.

Complex III activity is greatly reduced in the crystalline inclusion containing mitochondria compared to control mitochondria. The globular crystalline inclusions are not labeled and mitochondria with whirl shaped cristae are also lightly labeled for complex III antibody in this study.

These results suggest that the ultrastructurally changed inner membranes of mitochondria cause a loss in the activity of complex III. In addition, the mitochondria containing the globular crystalline inclusions are only labeled on the cristae which is similar to the control mitochondria. This indicates that complex III activity is present in the mitochondria except in the region of the globular paracrystalline inclusion. Although it has been suggested that the crystalline inclusions are proteins of subunits of the respiratory chain enzymes(Farrants *et al.*, 1986), the globular crystalline inclusion and whirl shaped cristae do not react to complexes I, III and IV antibody.

These results show that the globular crystalline inclusions are not related to the respiratory enzymes exsisting in the inner membrane of the mitochondria.

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미토콘드리아內 結晶含有物의 미세구조 및 免疫黃金標識法

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미토콘드리아가 포함하고있는 結晶含有物의 미세구조와 免疫黃金標識法에 의한 分析을 위하여 牛心筋 細胞의 미토콘드리아에서 電子傳達系에 관여하는 효소를 分離하였다. 牛心筋 미토콘드리아에서 分離된 효소는 실험토끼에 주사하여 電子傳達系 酵素(복합체 I, NADH-coenzyme Q reductase; 복합체 III, Ubiquinol-cytochrome-c-oxidoreductase; 복합체 IV, Cytochrome-c-oxidase)들에 대한 면역항체를 얻었다. 이들 면역항체들은 牛心筋과 正常人 骨格筋 미토콘드리아와 미토콘드리아에 結晶含有物을 포함하는 mitochondrial myopathy환자의 骨格筋 미토콘드리아에 면역반응시켜 黃金粒子를 標識하고 電子顯微鏡을 이용하여 이들 免疫抗體反應을 관찰하였다.

미토콘드리아가 포함하는 結晶含有物의 미세구조에는 paracrystalline inclusion body와 multilamellar structure inclusion body 그리고 球形結晶含有物(globular crystalline inclusion body) 및 輪形構造(whirl shaped structure)의 크리스테 中心에 있는 球形結晶含有物 등의 4종류로 관찰되었다.

복합체 I, 복합체 IV의 효소에 대한 항체를 牛心筋과 正常人 骨格筋 그리고 mitochondrial myopathy환자의 骨格筋에 동일한 면역반응을 시켰을 때 미토콘드리아 크리스테에 부착하는 黃金粒子의 標識 정도는 각각의 筋組織에서 유사한 反應이 관찰되었다.

복합체 III의 효소에 대한 항체는 牛心筋과 正常人의 骨格筋에서는 유사한 반응이 나타났으나 mitochondria myopathy환자의 골격근에서는 극히 소수의 黃金粒子가 관찰되었다.

球形結晶含有物은 복합체 I, III, IV의 3종류의 효소에 대한 면역반응 결과 黃金粒子標識은 관찰되지 않았다.

따라서 mitochondrial myopathy환자의 미토콘드리아에는 복합체 III의 효소가 缺乏되었으며 球形結晶含有物은 電子傳達系 효소들인 복합체 I, III, IV 酵素蛋白質과는 상관없는 物質로 생각된다.

Legends to Figures

Fig. 1. The electron dense enlarged cristae is composed of the inner memberane of mitochondria and a lot of glycogen accumulates in the sarcoplasm of the muscle of the mitochondrial myopathy patient tissue. (x 35,000)

Figs. 2, 3. The mitochondria containing the paracrystalline inclusion(PC) are elongated in length and the paracrystaline inclusion is made up of several multilamellar structures and is densely packed. (fig. 2, x 85,000; 3, x 56,000)

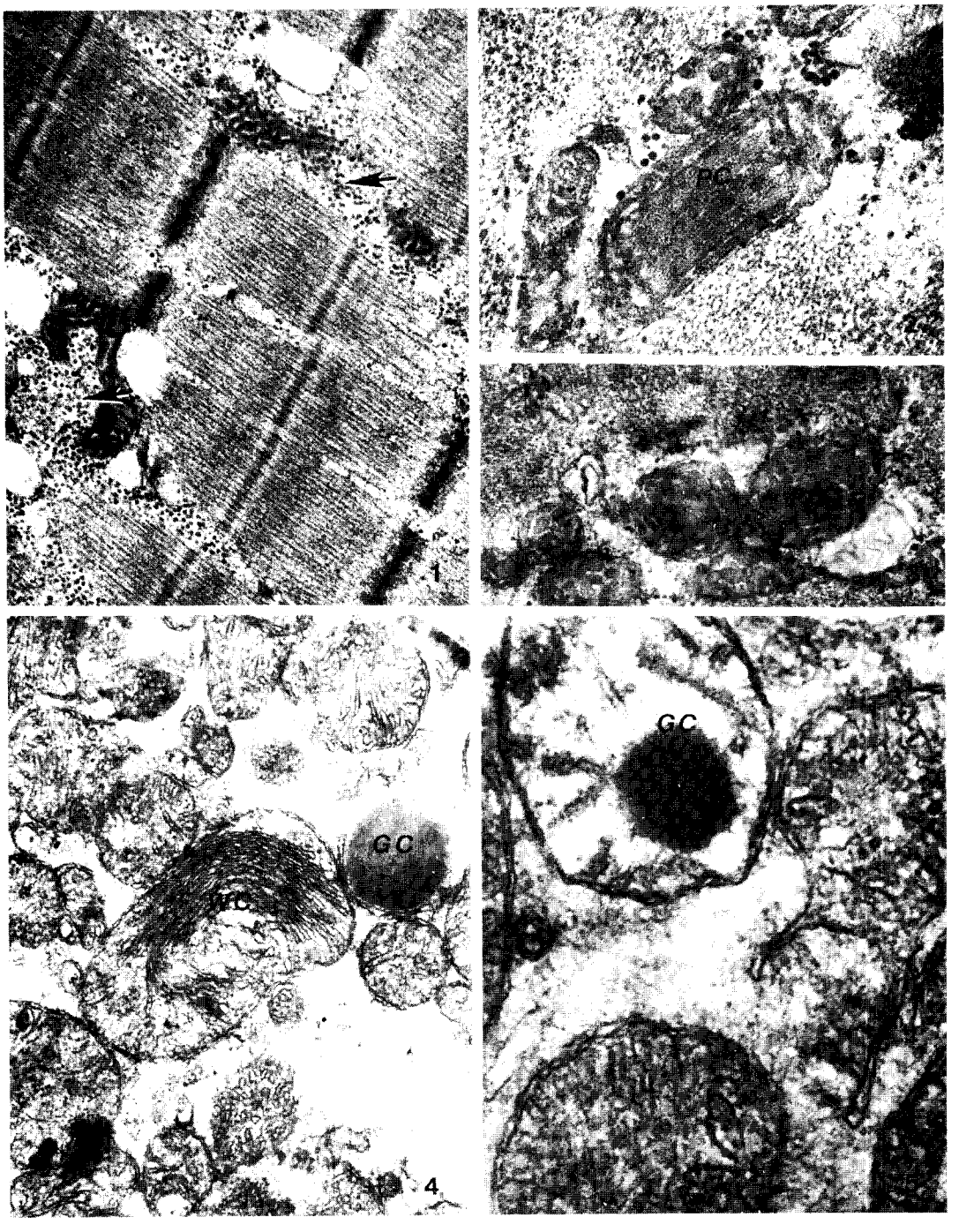
Fig. 4. A few of mitochondria have irregularly concentrated whirl shape (WC) cristae and are larger than the other mitochondria. (x 55,000)

Fig. 5. The globular crystalline(GC) inclusions which are very electron dense usually appear in normally sized mitochondria which remarkably have a few residual cristae or degenerated cristae. x 104,000

Figs. 6, 8, 10. Electron micrographs of a section of beef heart tissue, labeled with antibodies against complex I(Fig. 6), complex III(Fig. 8) and complex IV(Fig. 10), followed by protein A colloidal gold 12 nm particles. (x 30,000)

Figs. 7, 9, 11. Electron micrographs of a section of human skeletal muscle tissue, labeled with antibodies against complex I(Fig. 7), complex III(Fig. 9) and complex IV(Fig. 11), followed by protein A colloidal gold 12 nm particles. (x 30,000)

Figs. 12, 13, 14, 15. Electron micrographs of sections of myopathy patient muscle mitochondria containing globular crystalline(GC) inclusions, labeled with antibodies against complex I(Fig. 12), complex III(Figs. 13 & 14) and complex IV(Fig. 15)(fig. 12, x 48,000; 13, x 60,000, 14, x 38,000; 15, x 38,000)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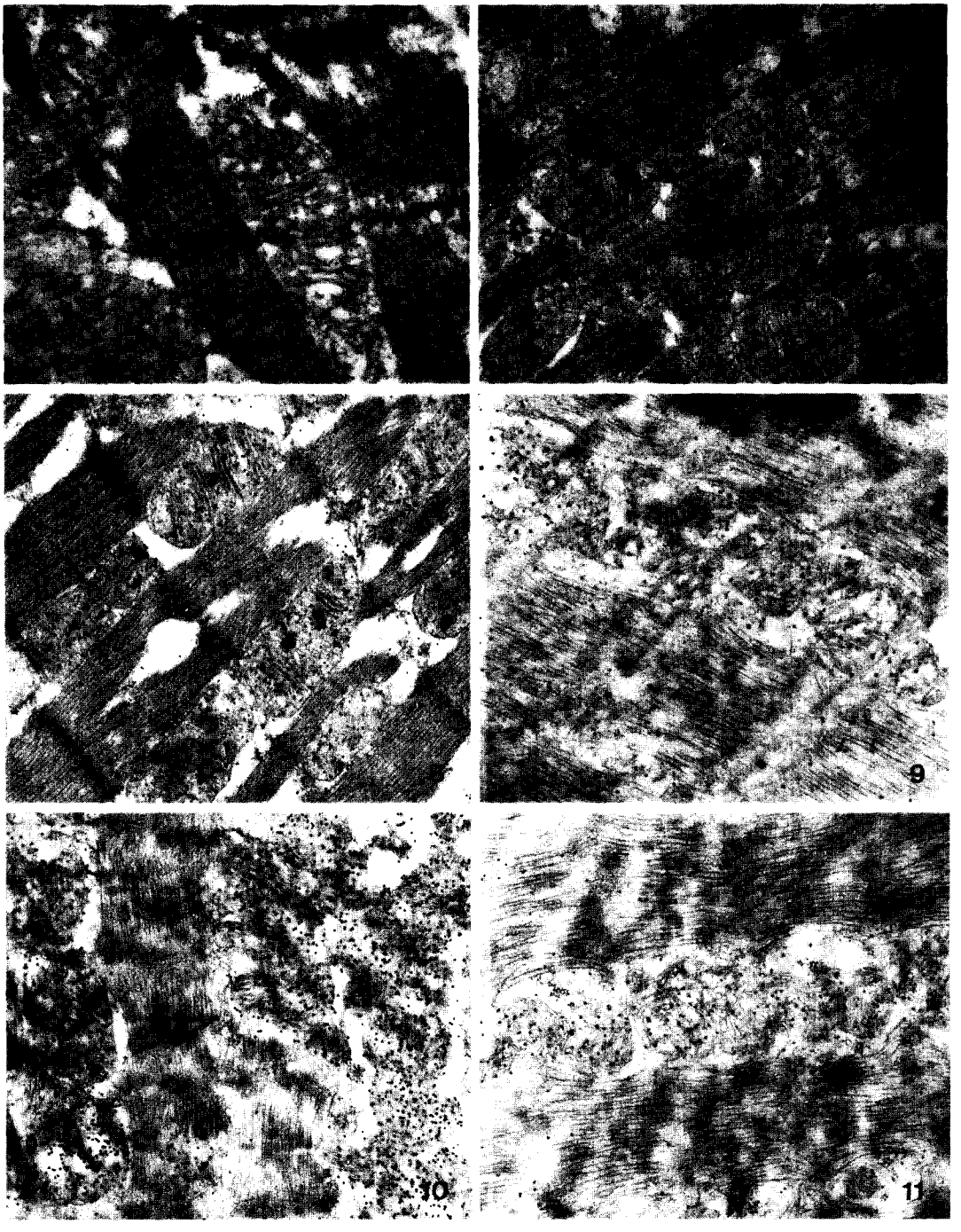
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